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Morphological Variants of Leukemic Cells in B Chronic Lymphocytic Leukemia Are Associated With Different T Cell and NK Cell Abnormalities

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B chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease. The different morphological variants of leukemic B cells appear to define different clinical groups of patients. Several abnormalities have been found in T lymphocytes and natural killer (NK) cells from B-CLL patients. We have investigated the phenotypic and functional characteristics of purified CD2+ cells from B-CLL patients at Binet's stage A and classified according to the neoplastic B lymphocyte morphology criteria: 32 patients with typical B-CLL and 12 patients with atypical B-CLL. Forty-three age and sex matched healthy controls were also studied. In fresh purified CD2+ cells from typical B-CLL patients, percentages of CD4+, CD4+CD45RA+, CD8+CD45RA+ T lymphocytes and CD3−CD56+ (NK) cells were significantly higher than those found in atypical B-CLL patients. However, in DC2+ cells from typical B-CLL patients, percentages of CD3+, CD3+DR+, CD8+, CD4+CD45RO+, and CD3+CD56+ cells were significantly lower than those found in atypical B-CLL patients. Increased percentage of NK cells was only found in typical B-CLL patients. The proliferative response and the production of interleukin (IL)-2 and IL-4 by phytohemagglutinin (PHA) stimulated CD2+ cells were significantly higher in typical B-CLL patients than in atypical B-CLL patients. We concluded that different patterns of phenotypic and functional alterations in the T lymphocytes and NK cells of B-CLL patients are found in patients with typical or atypical B-CLL defined according to the morphology of the leukemic cells. Am. J. Hematol. 55:175–182, 1997. © 1997 Wiley-Liss, Inc.

Key words: B chronic lymphocytic leukemia; cytotoxic T lymphocytes; NK cells; lymphocyte morphology

INTRODUCTION

B chronic lymphocytic leukemia (B-CLL) is a lymphoproliferative syndrome characterized by the proliferation and accumulation of malignant CD5+ B lymphocytes [1–4]. Highly variable clinical courses have been found in B-CLL patients [2]. An understanding of the factors involved in pathogenesis of this variability might allow identification of different patient subsets with potential therapeutic management. Comprehension of B-CLL requires the simultaneous analysis of leukemic cells and non-tumor cells. Along these lines, it has been observed that leukemic B cells from different B-CLL patients show heterogeneous morphological, phenotypical, and functional characteristics [5–7]. The morphological heterogeneity of the leukemic B cells has led to attempts to classify B-CLL patients into different morphological variants [8,9]. The prognostic value of morphological variability has been analyzed in several studies [10–12]. According to the French-American-British (FAB) group

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TABLE I. Morphologycal Variants of Leukemic Cells in B-CLL

Neoplastic cell morphology (size of the sample)	Circulating small cells (%)[a]	Circulating large cells (%)[b]	Circulating prolymphocytes (%)[c]	Other features
Typical CLL (n = 32)	>85	<15	>10	—
Atypical CLL (n = 12)	<85	>15	<10	Cleaved nucleus
CLL/PL (n = 0)	Variable	Variable	11–54	—

[a]Defined as lymphocytes with cell size <14 μm, clumped chromatin, nucleolus absent, and scanty cytoplasm.
[b]Defined as lymphocytes with cell size >14 μm, clumped chromatin, nucleolus absent or very small and poorly defined, and cytoplasm relatively abundant.
[c]Defined as lymphocytes with cells size >14 μm, clumped chromatin, nucleolus central and prominent, and variable amount of cytoplasm.

on B-CLL, criteria have been proposed 3 morphological types based on the proportion of atypical lymphocytes in blood of B-CLL: typical CLL, atypical CLL, and CLL/prolymphocytic leukemia (CLL/PL) [4]. An increase in the percentage of large leukemic B lymphocytes is usually associated with poorer prognosis and shorter survival times [13].

Neoplastic cells from solid tumor or leukemic patients establish several functional interactions with the host immune system [14,15]. The immune system response to the neoplasm may have either defensive significance for the host or, in contrast, may constitute a mechanism of promotion of growth and dissemination of tumor cells [14]. The lymphocytes with potential ability to respond to leukemic cells in B-CLL patients include T lymphocytes and natural killer (NK) cells. Several phenotypic and functional alterations in T lymphocytes [16–21] and NK cells [22,23] have been described in B-CLL patients. It has been shown that the total number of T lymphocytes and NK cells in the peripheral blood is increased [24]. Abnormal distribution of T cell subsets, as defined by the expression of the surface antigens CD4, CD8 [18–20,25,26], CD45RA [20,27,28], CD45RO [29], CD56 and CD57 [24] have been described. T lymphocytes from these patients also show increased expression of activation antigens such as CD25 and the HLA system class II molecules [27,30]. Defective activation and proliferative responses to mitogens and impaired cytotoxic activity and abnormal patterns of cytokine production have also been observed in T lymphocytes from B-CLL patients [16,18,21,23]. NK cells also show defective cytotoxic activity in these patients [22,23]. The cause and pathogenic signification of these findings remain unknown. Presently these T lymphocyte and NK cell abnormalities are found to be distributed in a heterogeneous pattern in B-CLL patients.

It is know that differences in the morphological characteristics of leukemia cells are associated with different expressions of structural and functional proteins, including cytokines and potential antigens [5]. It has also been claimed that distinct B-CLL leukemic cell subsets show different activation and growth requirements [6,8]. Along these lines, it is possible to hypothesize that different abnormalities in the non-tumoral T lymphocyte and NK compartments could be associated with different morphological patterns of leukemia B cells found in patients.

This paper analyzes the possible relationships between the morphological characteristics of neoplasic cells and the different characteristics (phenotypical and functional) of T and NK cell compartments from B-CLL patients.

MATERIAL AND METHODS

Patient Populations

Forty-four patients with B-CLL (19 women, 25 men, mean age 69 years, range 44–85) diagnosed between 1989 and 1995 have been included in this study. The diagnosis was based on morphological and immunological features [31]. Clinical staging was performed according to Binet et al. [32]. All patients belonged to stage A. Patients showed no evidence of current acute or chronic disease other than B-CLL and no history of pathological conditions, which might have affected the immune system, or alterations in their nutritional state. Further, the B-CLL patients were classified according to their leukemic B cell morphology as described by Matutes et al. [5] (Table I) in two groups: B-CLL typical (12 women, 20 men, mean age 71, range 44–85, median lymphocyte count 18.5×10^9/l, range 3.4–46.2) and B-CLL atypical (5 women, 7 men, median age 76, range 71–81, median lymphocyte count 17.2×10^9/l, range 6.5–35.0). We have not found any case of CLL/PL, PL, or atypical CLL with lymphoplasmocytic features. Morphology of neoplastic cells was restudied every 3 months and none of the patients studied showed morphological transformation during all post-experimental follow-up. Forty-three age and sex matched normal, voluntary blood donors were used as healthy controls. All were asymptomatic and reported no B-CLL disease in their pre-donation interviews. Neither patient nor control group subjects took drugs known to affect the immune system.

Morphology

Peripheral blood and bone marrow films were made on glass slides and stained according to the May-Grünwald-Giemsa method. B cell morphology was determined by microscope examination, and reviewed by a panel of three senior hematologists and a minimum of 500 lymphocytes was evaluated. In 3 cases of disagreement on cell morphology, the films were reviewed until a consensus was reached. The criteria used to define the percentage of small cells, large cells, and prolymphocytes are shown in Table I [33].

Cell Separation

Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation in a Ficoll-Hypaque (Lymphoprep Nyegaard and Co., Oslo, Norway) density gradient as previously described [34]. T cells were purified by double rosetting with sheep red blood cells (SRBCs) pretreated with a 2-aminoiethylisothio-uronium bromide (AET) gradient as previously described [35]. Rosetted lymphocytes were freed of SRBCs by brief hypotonic lysis. After counting, cells were resuspended in RPMI 1640 (Whitaker Bioproducts, Walkersville,) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom KG, Berlin, Germany), 2 mM L-glutamine (Biochrom KG), 25 mM Hepes (Biochrom KG) and 1% penicillin-streptomycin (Difco Lab, Detroit, MI). This will be referred to as complete medium (CM). Cell viability, as checked by trypan blue exclusion, was always greater than 95%. The purity of phenotypically defined CD2+ lymphocytes in the rosetted cell preparations was, in all cases, greater than 90%.

Staining and FACS Analysis

For immunofluorescence, fresh T lymphocytes were incubated with combinations of fluorescein isotiocianate (FITC, green), phycoerythrin (PE, orange), and peridinin chlorophyll protein conjugate (PerCP, red)-labeled monoclonal antibodies (MoAbs). These were used in three-color combinations to define the enriched T cell preparations and activated cells (FITC/PE/PerCP) in each tube: Tube 1, anti-CD19 (B cells)/anti-CD2 (all T cells and NK cells)/anti-CD3 (all T cells); Tube 2, anti-CD19/anti-CD56 (N-CAM, defines NK cells as CD3-CD56+ and also non-MHC restricted T lymphocytes CD3+CD56+)/anti-CD3; Tube 3, anti-HLA-DR (MHC class II molecules)/anti-CD25 (p55, α chain of IL-2R)/anti-CD3; Tube 4, anti-CD45RA (unprimed T cells)/anti-CD45RO (primed T cells)/anti-CD4 (MHC class II restricted T lymphocytes); Tube 5, anti-CD45RA/anti-CD45RO/anti-CD8 (MHC class I restricted T lymphocytes). Control studies with unstained cells and cells incubated with isotype-matched irrelevant FITC-, PE-, and PerCP-labeled monoclonal antibodies were performed for each experiment. All MoAbs were obtained from Becton Dickinson (Mountain View, CA). Tri-color immunofluorescent analysis was performed with a FACScan flow cytometer and Lysis II software (Becton Dickinson). A biparametric gate in the FSC-SSC dot plot was drawn around the lymphocyte population defined by an antigen expression of CD45+CD14−.

Proliferation Studies

Fresh CD2+ lymphocytes (50,000 cells/well) were cultured in CM on 96-well, flat-bottomed microtiter plates (Nunc Corporation, Roskilde, Denmark) in the presence or absence of 10 μg/ml phytohemagglutinin (PHA) (Difco Lab). This reagent was tested in dose/response titrations before use. Cultures were incubated at 37°C in a 95% humid atmosphere containing 5% CO_2 for 5 days. DNA synthesis was measured during the last 18 h of the culture period by incorporation of [^{3}H] thymidine (Radiochemical Center, Amersham, UK). Cultures were performed in triplicate and the standard deviation between replicates was < 10%.

Measurement of IL-2 and IL-4 Productions

To measure production of IL-2 and IL-4, 5×10^6 fresh CD2+ lymphocytes from B-CLL patients and healthy controls were cultured in 1 ml of CM in the presence or absence of 10 μg/ml PHA (Difco Lab). Cell cultures were maintained for 72 h in an incubator at 37°C in a 95% humid atmosphere containing 5% CO_2. The culture supernatants were then harvested, sterilized by filtration through an 0.22-μm filter (Millipore Company, Bedford, CA), separated into aliquots and quickly stored at −70°C until use. Cytokine levels in cell culture supernatants were determined in duplicate using the commercially available specific IL-2 ELISA kit (T Cell Diagnostics, Cambridge, MA) and the IL-4 ELISA kit (Genzyme Co., Boston, MA). Each data point is the average of two determinations, neither of which varied within 10% of the final average. Results are expressed in pg/ml. The sensitivity of the IL-2 and IL-4 Test Kits was 59 and 45 pg/ml, respectively.

Statistical Analysis

Data from groups were compared with the Mann-Whitney U-test. *P* values were considered significant where less than 0.05.

RESULTS

Different Distributions of T Lymphocyte and NK Cell Subsets Are Found in B-CLL Patients With Typical or Atypical Leukemic Cell Morphology

The 44 B-CLL patients at Binet's stage A included in this study were classified according to their leukemic B cell morphology [5]. There were no significant differ-

TABLE II. Immunophenotyping of CD2+ Fractions From B-CLL and Healthy Controls

Marker	Healthy controls (n = 43)	Typical B-CLL patients (n = 32)	Atypical B-CLL patients (n = 12)
CD3+	87.5 ± 5.7	80.5 ± 10.0*	87.8 ± 5.1****
CD3+CD25+	4.5 ± 2.0	8.4 ± 4.7**	10.1 ± 4.1**
CD3+HLADR+	6.7 ± 3.6	19.7 ± 2.3*	28.3 ± 6.0*·*****
CD4+	53.0 ± 10.1	47.2 ± 11.9***	39.4 ± 15.3**·*****
CD8+	36.5 ± 11.1	36.8 ± 13.1	48.8 ± 15.6**·****
CD4/CD8 ratio	1.64 ± 0.85	1.56 ± 1.03	0.95 ± 0.58***·*****
CD4+CD45RA+	31.0 ± 10.3	22.4 ± 11.0***	6.4 ± 2.8**·****
CD4+CD45RO+	20.4 ± 6.5	23.0 ± 9.4	32.4 ± 7.6**·*****
CD8+CD45RA+	24.3 ± 6.8	24.0 ± 10.1	16.8 ± 8.2***·*****
CD8+CD45RO+	12.9 ± 5.3	12.7 ± 7.3	31.8 ± 9.2**·****
CD3−CD56+	9.8 ± 5.3	15.7 ± 9.3**	7.6 ± 3.4****
CD3+CD56+	6.5 ± 4.2	8.5 ± 1.2	15.1 ± 5.1***·****

†The patients were classified according to leukemic B cell morphology. Values represent the mean ± SD.
*$P < 0.001$ vs. healthy controls.
**$P < 0.01$ vs. healthy controls.
***$P < 0.05$ vs. healthy controls.
****$P < 0.01$ vs. typical B-CLL patients.
*****$P < 0.05$ vs. typical B-CLL patients.

ences in the male/female ratio, age, and median peripheral blood lymphocyte counts between the group of 32 patients with typical B-CLL and the other group of patients with atypical B-CLL (data not shown). Forty-three age and sex matched healthy donors were use as controls. The phenotypical characteristics of both groups of the T lymphocyte and NK cell subsets in the purified CD2+ lymphocyte preparations from both groups of patients and healthy controls were analyzed. There were no significant differences in the percentage of purified CD2+ lymphocytes from typical and atypical B-CLL groups and healthy controls (median and interquartile range: 97.8 [95.4–99.8], 96.2 [93.7–98.8], and 98.6 [95.8–99.4], respectively). The percentage of CD3+ lymphocytes in the CD2+ cell fractions from typical B-CLL patients was significantly lower than that found in either atypical B-CLL or healthy controls ($P < 0.01$; $P < 0.001$, respectively), but there were no significant differences between the two last groups (Table II).

CD25 and HLA-DR expression by T lymphocytes from both groups of B-CLL were significantly higher than those of healthy controls ($P < 0.01$; $P < 0.001$, respectively). There were no significant differences in the percentages of CD25+ T lymphocytes in the cellular fractions between both groups of B-CLL patients. However, the expression of the activation antigen DR on T lymphocytes in typical B-CLL patients was significantly lower than that of atypical B-CLL patients ($P < 0.05$) (Table II).

As seen in Table II, the percentage of CD4+ lymphocytes in the CD2+ cell fractions from typical B-CLL and atypical B-CLL patients was significantly lower than that found in healthy controls ($P < 0.05$; $P < 0.01$, respectively). Interestingly, the percentage of CD4+ lymphocytes in typical B-CLL patients was significantly higher than that found in atypical B-CLL patients ($P < 0.05$). Simultaneously, the percentage of CD8+ lymphocyte in atypical B-CLL patients was significantly higher than that found in typical B-CLL patients and healthy controls ($P < 0.01$). The percentage of CD8+ lymphocytes in purified CD2+ cell fractions from typical B-CLL patients and healthy controls as similar. The ratio CD4/CD8 in atypical B-CLL patients was significantly higher than that found in typical B-CLL patients and healthy controls ($P < 0.05$ in both cases).

The expression of surface antigens associated with T lymphocyte antigen-priming in purified CD2+ cell fractions from both patient groups and healthy controls was also studied (Table II). The percentages of CD4+ CD45RA+ in CD2+ purified fractions from typical B-CLL and atypical B-CLL patients were significantly lower than that found in healthy controls ($P < 0.05$; $P < 0.01$, respectively). The percentages of CD4+CD45RA+ in typical B-CLL patients were significantly higher than in atypical B-CLL patients ($P < 0.01$). The percentages of CD4+CD45RO+ in cellular preparations from atypical B-CLL patients were significantly higher compared to typical B-CLL patients and healthy controls ($P < 0.05$, $P < 0.01$, respectively). There were no significant differences between the percentage of CD4+CD45RO+ cells from CD2+ cells present in the cellular preparations and typical B-CLL patients and healthy controls. The percentages of CD8+CD45RA+ and CD8+CD45RO+ lymphocytes in the cellular preparations from typical B-CLL patients were similar to those of healthy controls (Table II). The percentage of CD8+CD45RA+ cells from atypical B-CLL patients was significantly lower than that measured in typical B-CLL patients and healthy controls ($P < 0.05$). In addition, the percentage of CD8+ CD45RO+ lymphocytes in the cellular preparations from atypical B-CLL patients was significantly higher than that of typical B-CLL patients and healthy controls ($P < 0.01$).

The expression of the antigen CD56, associated with lymphocyte cytotoxic functions, was also analyzed in the purified CD2+ cell fractions from B-CLL patients and healthy controls (Table II). The percentage of NK cells (CD3−CD56+) in typical B-CLL patients was significantly higher than those in atypical B-CLL patients and healthy controls ($P < 0.01$). There were no statistical differences between the percentages of NK cells (CD3−CD56+) in atypical B-CLL patients and healthy controls. The percentage of potential cytotoxic T lymphocytes (CD3+CD56+) (CTL) in atypical B-CLL patients was significantly higher than those in typical B-CLL patients and healthy controls ($P < 0.01$, $P < 0.05$,

TABLE III. Proliferative Response and IL-2 and IL-4 Production by PHA Activated CD2+ Lymphocytes From B-CLL Patients and Healthy Controls†

Functional characteristics	Healthy controls (n = 43)	Typical B-CLL patients (n = 32)	Atypical B-CLL patients (n = 12)
Proliferative response (cpm × 10^3)	137 ± 68	95 ± 61***	51 ± 26*,****
Cytokine production (pg/ml)			
IL-2	273 ± 52	913 ± 246**	119 ± 34*,****
IL-4	165 ± 91	739 ± 166**	349 ± 94

†The proliferative response (expressed as cpm) and cytokine production (expressed as pg/ml) by CD2+ cells purified from B-CLL patients and healthy controls were determinated as described in Materials and Methods. The patients were classified according to leukemic B cell morphology. Values represent the mean ± SD or lower limit detection of ELISA kit.
*$P < 0.001$ vs. healthy controls.
**$P < 0.01$ vs. healthy controls.
***$P < 0.05$ vs. healthy controls.
****$P < 0.01$ vs. typical B-CLL patients.

respectively). There were no statistical differences between the percentage of potential cytotoxic T lymphocytes (CD3+CD56+) cells in typical B-CLL patients and healthy controls.

Although the differences in the distribution of age between both groups of patients were not significant, all atypical B-CLL patients are older than 71 years. In order to exclude that aging could bias the data obtained in the group of typical B-CLL patients, a comparative analysis was performed between 71 older (n = 16) or younger (n = 16) patients. There were no significant differences in the different phenotypical parameters analyzed in CD2+ preparations between both age groups of typical B-CLL patients (data not shown).

Functional Characteristics of CD2+ Cells: Their Correlation With Morphological Features

The PHA induced proliferative responses of CD2+ purified cell populations from both groups of B-CLL patients and healthy controls were also analyzed (Table III). In kinetic studies performed at 3, 5, and 7 days of culture, the maximal proliferative response to PHA of purified CD2+ preparations from 8 B-CLL patients (4 typical B-CLL and 4 atypical B-CLL) and 6 healthy controls, was found at 5 days of culture (data not shown). Thus, cultures at 5 days were routinely analyzed in all experiments performed. It was found that the proliferative responses to PHA of CD2+ lymphocytes from typical and atypical B-CLL patients were significantly lower than that found in healthy controls ($P < 0.05$, $P < 0.001$, respectively). The blastogenic response of PHA-stimulated purified CD2+ preparations was significantly higher in typical B-CLL patients than that found in atypical B-CLL patients ($P < 0.01$).

The IL-2 and IL-4 production by PHA-stimulated CD2+ cells from both groups of B-CLL patients and healthy controls was also analyzed. The production of IL-2 and IL-4 in PHA-stimulated CD2+ cell fractions from typical B-CLL patients was significantly higher than that found in healthy controls ($P < 0.01$). IL-2 production by PHA-stimulated CD2+ cells from atypical B-CLL patients was significantly lower than that found in typical B-CLL patients and healthy controls ($P < 0.01$). However, IL-4 production by CD2+ cells from atypical B-CLL patients was similar to that found in healthy controls. The IL-2 and IL-4 production by non-stimulated CD2+ cells from B-CLL patients and healthy controls was under the limit of detection of the ELISA (data not shown).

DISCUSSION

In this work, we have found a clear association between different patterns of phenotypical and functional abnormalities in T lymphocyte and NK cell compartments of B-CLL patients and the morphological characteristics of the leukemic cells. We have observed that some of the alterations previously reported to be heterogeneously found in B-CLL patients such as a decrease in CD4/CD8 ratio, increase in the percentage of activated T cells, and decreased proliferative response of CD2+ cells to polyclonal mitogens are more profound in atypical B-CLL whereas other alterations, like an increase in NK cells, are only found in typical B-CLL patients.

It has been described that T lymphocytes from B-CLL patients show an abnormal distribution of subsets as defined by the expression of the CD4 and CD8 antigens [28]. A decreased percentage of CD4+ T lymphocytes and normal or increased percentage of CD8+ lymphocytes in purified CD2+ populations from these patients have been observed [23–28,36]. In this study, the distribution of the CD4 and CD8 subsets in purified CD2+ populations from typical B-CLL and atypical B-CLL patients were seen to be markedly different. In atypical B-CLL patients, a clear expansion of the CD8+ T lymphocyte subset and a concomitant reduction of the CD4+ subset was found. In contrast, in typical B-CLL patients, the percentage of CD8+ lymphocytes is similar to that found in healthy controls and the reduction in the percentage of CD4+ lymphocytes is notably less marked that in atypical B-CLL patients. A clear probe of these differences is that the CD4/CD8 ratio was twofold lower in atypical B-CLL patients than in typical B-CLL patients and healthy controls.

The expression of the CD45 isoform has been associated with different stages of T lymphocyte antigen priming [37]. The CD45RO+ T lymphocytes represent antigen driven cells, whereas antigen unprimed T lymphocytes are CD45RA+ cells [37]. The present data show the existence of a clear shift towards the expression of the

CD45RO isoform in both CD4 and CD8 T lymphocyte subsets of atypical B-CLL patients. A concomitant reduction in the percentage of the CD4+CD45RA+ and CD8+CD45RA+ T cells is observed in subjects with atypical morphology. In contrast, T lymphocytes from typical B-CLL patients did not show preferential expression of the CD45RO isoform. A similar expression of the RO and RA isoforms of the CD45 antigen in the CD8 T subset was found in typical B-CLL patients as that found in healthy controls. However, a reduced percentage of CD45RA cells was found in the CD4+ lymphocytes from typical B-CLL patients. The expression of class II molecules by T lymphocytes is considered to be a phenotypic event of cellular activation. The T lymphocyte compartment of atypical B-CLL patients not only shows a shift towards the expression of the RO isoform but also to an enhanced expression of DR molecules. It is important to remember that the previously described decrease in the percentage of CD4+CD45RA+ cells [26] is mainly observed in the atypical B-CLL group. This marked difference in the distribution of antigen driven lymphocyte subsets and activation antigen expression in T lymphocytes, from both atypical B-CLL and typical B-CLL patients, may explain the different results previously published in works on the phenotypic characteristics of T lymphocytes in B-CLL patients [21,38]. Preferential inclusion of patients with a morphologically defined pattern of leukemic B cells represent a bias that affects the results in the phenotypic analysis of non-tumoral T lymphocytes of B-CLL patients.

Though it has been reported that the percentage of NK cells (CD3−CD56+) in the CD2+ cells of B-CLL is increased [29,39], our data demonstrate that only typical B-CLL patients show raised percentages of NK cells (CD3−CD56+). However, this conclusion is softened by the small numbers of atypical B-CLL patients. Since these NK cells are CD2+ but not CD3−, their significant relative increase in typical B-CLL patients explains the decrease in CD3+ lymphocytes within CD2+ cell fractions from these patients. However, in atypical B-CLL patients, the percentage of NK cells was not increased. Nevertheless, in this latter group, an increased percentage of CD3+CD56+ T lymphocytes was observed. These results reveal that phenotypic analysis of the expression of single antigens in different lymphocyte subsets in B-CLL peripheral blood, may give confusing and even misleading results.

It has been reported that the proliferative response of T lymphocytes to polyclonal mitogens is heterogeneous in B-CLL patients [16,21,40]. We studied the functional behavior of purified CD2+ cells from both typical and atypical B-CLL patients. We have found that the proliferative response of CD2+ cells to PHA is defective in both groups of B-CLL patients as compared to healthy controls. However, the PHA stimulated blastogenic response of CD2+ cells from typical B-CLL patients was higher than that of atypical B-CLL patients. This different degree of impairment of the blastogenic response of CD2+ cells to T lymphocyte mitogen stimuli, may also explain the different proliferative responses previously reported [21]. It is possible that the lower degree of proliferative response found in CD2+ cells from atypical B-CLL patients might be related to their higher degree of T lymphocyte in vivo activation and their T lymphocyte subset redistribution.

It is known that cytokine-secreting T lymphocytes constitute a heterogeneous population [37,40,41]. It has been reported that different T lymphocyte subsets can produce IL-2 and IL-4 [6]. The preferential production of IL-2 or IL-4 by T lymphocytes has been used for the recognition of the Th1 and Th2 functional subsets, respectively [42]. Therefore, an analysis was made of the production of both cytokines by PHA-activated CD2+ cells from both typical and atypical B-CLL patients. CD2+ cells from these patient groups showed different levels of production of IL-2 and IL-4 after PHA stimulation. Increased levels of these cytokines were found in supernatants of CD2+ PHA-stimulated cells from typical B-CLL patients. In contrast, impaired IL-2 and normal IL-4 production were found in supernatants of CD2+ PHA-stimulated cells from atypical B-CLL patients. It is known that the CD4+ and CD8+ T lymphocyte populations, as well as their subsets, defined by the preferential expression of the CD45 isoforms RA and RO, show different behavior in activation and proliferation requirements as well as in cytokine secretion [6]. Therefore, the different T cell distributions of typical B-CLL and atypical B-CLL patients might be involved in the different functional behavior of the CD2+ cells observed in these subjects.

According to the current biological and clinical knowledge, B-CLL may be considered to be a defined lymphoproliferative syndrome including several different disease [7,43–45]. Unfortunately, the causes of this heterogeneity in B-CLL patients remain elusive. Our data clearly show that typical B-CLL and atypical B-CLL patients have markedly different phenotypical and functional abnormalities in their T lymphocyte and NK compartments. It is possible that differences in the intrinsic characteristics of leukemic cells and/or the patterns of alterations of T lymphocyte and NK cells may constitute the biological bases of their clinical heterogeneity.

Several mechanisms may be involved in the different patterns of alterations in the T lymphocyte and NK cell compartments found in both groups of B-CLL patients. These two patterns of abnormalities in T and NK cells may represent the passive effects and/or active immune response to both types of leukemic B cells. The different morphological characteristics of typical and atypical leukemic cells may also be associated with potential anti-

genic and/or functional differences [5,46]. The existence of activated T lymphocytes with an increased percentage of antigen driven cells and the described preferential use of T cell receptor β chain rearrangement genes in B-CLL patients, is in agreement with this possibility [36,47]. Another explanation is that the different abnormal backgrounds of T lymphocytes and NK cells might allow the preferential expansion and accumulation of different leukemic B cells. It is know that primary or acquired immunodeficiencies appear to be associated with different tumors [48,49]. Our results indicate that the biological understanding of B-CLL requires the simultaneous study of neoplasic B cells and host non-tumor transformed immune system.

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REFERENCES

1. Galton DAG: The pathogenesis of chronic lymphocytic leukemia. Can Med Ass J 94:1005, 1966.
2. Boogs DR, Sofferman SA, Wintrobe MM, Cartwright GE: Factors influencing the duration of survival of patients with chronic lymphocytic leukemia. Am J Med 40:243, 1966.
3. Damesheck W: Chronic lymphocytic leukemia-an accumulative disease of immunologically incompetent lymphocytes. Blood 29:566, 1967.
4. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: The French-American-British (FAB) Cooperative Group proposals for the classification of chronic (mature) B and T lymphoid leukaemias. J Clin Pathol 42:657, 1989.
5. Matutes E, Oscier D, Garcia-Marco J, Ellis J, Copplestone A, Gillingham R, Hamblin T, Lens D, Swansbury GJ, Catovsky D: Trisomy 12 defines a group of CLL with atypical morphology: Correlation between cytogenetic, clinical and laboratory features in 544 patients. Br J Haematol 92:382, 1996.
6. Moreau JL, Chastagner P, Tanaka T, Miyasaka M, Kondo M, Sugamura K, Theze J: Control of the IL-2 responsiveness of B lymphocytes by IL-2 and IL-4. J Immunol 155:3401, 1995.
7. Baldini L, Mozzana R, Cesana B, Cortellezzi A, Neri A, Radaelli F: Clinical stage and immunological findings in chronic lymphocytic leukemia. Boll-Ist-Sieroter-Milan 63:50, 1984.
8. Jabbar SA, Hoffbrand AV, Gitendra-Wickremasinghe R: Regulation of transcription factors NK kappa B and AP-1 following tumor necrosis factor treatment of cells from chronic B cell leukaemia patients. Br J Haematol 86:496, 1994.
9. Melo JV, Catovsky D, Gregory WM, Galton DAG: The relationship between chronic lymphocytic leukaemia and prolymphocytic leukaemia. IV. Analysis of survival and prognostic features. Br J Haematol 65:23, 1987.
10. Dubner HN, Crowley JJ, Sciling RF: Prognostic value of nucleoli and cell size in chronic lymphocytic leukemia. Am J Hematol 4:337, 1978.
11. Peterson LC, Bloomfield CD, Brunning RD: Relationship of clinical staging and lymphocyte morphology to survival in chronic lymphocytic leukemia. Br J Haematol 54:562, 1980.
12. Ghani AM, Krause JR: Investigation of cell size and nuclear clefts as prognostic parameters in chronic lymphocytic leukemia. Cancer 58: 2233, 1986.
13. Litz CE, Bruning RD. Chronic lymphoproliferative disorders: Classification and diagnosis. Bailliere's Clin Haematol 1993.
14. Old LJ: Cancer immunology: The search for specificity. Cancer Res 41:361, 1981.
15. Grimm EA, Mazunder A, Zhang HZ, Rosemberg SA: Lymphokine-activated killer cell phenomenon. Lysis of natural killer resistant fresh solid tumour cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 155:1823, 1989.
16. Davis S: Characterization of the phytohemagglutinin-induced proliferating lymphocyte subpopulations in chronic lymphocytic leukemia patients using a clonogenic agar technique and monoclonal antibodies. Blood 58:1053, 1989.
17. Plastsoucas CD, Galinski M, Kempin S, Reich L, Clarkson B, Good RA: Abnormal T lymphocyte subpopulations in patients with B cell chronic lymphocytic leukemia: An analysis by monoclonal antibodies. J Immunol 129:2302, 1982.
18. Lauria F, Foa R, Montavani V, Fierro T, Catovsky D, Tura S: T-cell functional abnormality in B-chronic lymphocytic leukaemia: Evidence for a defect in the T-helper subset. Br J Haematol 54:277, 1983.
19. Kay NE, Kaplan ME: Defective expression of T cell antigens in chronic lymphocytic leukaemia: Relationship to T cell dysfunction. Br J Haematol 57:105, 1984.
20. Tötterman TH, Carlsson M, Simonsson B, Bengtsson M, Nilsson K: T-cell activation and subset patterns are altered in B-CLL and correlate with the stage of the disease. Blood 74:786, 1989.
21. Prieto A, Garcia-Suarez J, Reyes E, Lapeña P, Hernandez M, Alvarez-Mon M: Diminished DNA synthesis in T cells from B chronic lymphocytic leukemia after phytohemagglutinin, anti-CD3, and phorbol myristate acetate mitogenic signals. Exp Hematol 21:1563, 1993.
22. Ziegler HW, Kay NE, Zarling JM: Deficiency of natural killer cell activity in patients with chronic lymphocytic leukemia. Int J Cancer 27:321, 1981.
23. Alvarez de Mon M, Casas J, Laguna R, Toribio ML, de Landázury MO, Durantez A: Lymphokine induction of NK-like cytotoxicity in T cells from B-CLL. Blood 67:228, 1986.
24. Vuillier F, Tortevoye P, Binet JL, Dighiero G: CD4, CD8 and NK subsets in B-CLL. Nouv Rev Fr Hematol 30:331, 1988.
25. Herrmaun F, Lochner A, Philippen H, Jauer B, Ruhl H: Imbalance of T cell subpopulations in patients with chronic lymphocytic leukemia of the B cell type. Clin Exp Immunol 49:157, 1982.
26. Peller S, Kaufman S: Decreased CD45RA T cells in B-cell chronic lymphatic leukemia patients: Correlation with disease stage. Blood 78:1569, 1991.
27. Briggs PG, Kraft N, Atkins RC: T cells and CD45R expression in B-chronic lymphocytic leukemia. Leuk Res 14:155, 1990.
28. Dianzani U, Omedè P, Marmont F, DiFranco D, Fusaro A, Bregardo M, Redoglia V, Giaretta F, Mairone L, Boccadoro M, Resegotti L, Pileri A: Expansion of T cells expressing low CD4 or CD8 levels in B-cell chronic lymphocytic leukemia: Correlation with disease status and neoplastic phenotype. Blood 83:2198, 1994.
29. Frolova EA, Richards SJ, Jones RA, Rawstron A, Master PS, Teasdale J: Immunophenotypic and DNA genotypic analysis of T-cell and NK-cell subpopulations in patients with B-cell chronic lymphocytic leukemia (B-CLL). Leuk Lymphoma 16:307, 1995.
30. Terstappen LW, de Grooth BG, Segers-Nolten I, Greve J: Cytotoxic lymphocytes in B-cell chronic lymphocytic leukemia. A flow cyto-

metric study of peripheral blood, lymph nodes and bone marrow. Blut 60:81, 1990.
31. Garcia-Suarez J, Prieto A, Reyes E, Merino JL, Alvarez-Mon M: Increased percentage of activated T lymphocytes in B-cell lymphocytic leukaemia (B-CLL) patients. Br J Haematol 79:657, 1991.
32. Matutes E, Owusu-Ankomah K, Morilla R, Garcia-Marco J, Houlihan A, Que TH, Catovsky D: The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 8:1640, 1994.
33. Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, Jacquillat C, Boivin P, Lesty C, Duault MT, Monconduit M, Belabbes S, Gremy F: A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 48:198, 1981.
34. Melo JV, Catovsky D, Galton DAG: The relationship between chronic lymphocytic leukaemia and prolymphocytic leukaemia. I. Clinical and laboratory features of 300 patients and characterization of an intermediate group. Br J Haematol 63:377, 1986.
35. Böyum AJ: Isolation of mononuclear cells and granulocytes from human blood. Scan J Clin Lab Invest 21:77, 1968.
36. Madsen M, Johnsen HE, Hansen PW, Christiansen SE: Isolation of human T and B lymphocytes by E-rosette gradient centrifugation. Characterization of the isolated sub-populations. J Immunol Methods 33:323, 1980.
37. Farace F, Orlanducci F, Dietrich PY, Gaudin C, Angevin E, Coutier MH, Bayle C, Hercend T, Triebel F: T cell repertoire in patients with B chronic lymphocytic leukemia. J Immunol 143:4281, 1994.
38. Sanders ME, Makgoba MW, Shaw S: Human naive and memory T cells. Immunol Today 9:195, 1988.
39. Byrne JA, Butler JL, Cooper MD: Differential activation requirements for virgin and memory T cells. J Immunol 141:3249, 1988.
40. Antica M, Kusic B, Spaventi R, Jaksic B, Vitale B: Functional differences of T cells in B-chronic lymphocytic leukemia. Leuk Lymphoma 9:133, 1993.
41. Kalinski P, Hilkens CM, Wierenga EA, van-der-Pouw-Kraan TC, van-Lier RA, Bos JD, Kapsenberg ML, Snijdewint FG: Functional maturation of human naive T helper cells in the absence of accessory cells. Generation of IL-4-producing T helper cells does not require exogenous IL-4. J Immunol 154:3753, 1995.
42. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. Definition according to profiles of lymphokine activities and secretered proteins. J Immunol 136:2348, 1986.
43. Rai KR, Satwisky A, Cronkite EP, Chana AD, Levi RN, Pastermack BS: Clinical staging of chronic lymphocytic leukemia. Blood 46:219, 1975.
44. Mittelman A, Denny T, Gebhard D, Cirrincione C, Kurland E, Koziner B: Analysis of T-cell subsets in B-cell chronic lymphocytic leukemia: a correlation with the stage of disease. Am J Hematol 16:67, 1984.
45. Hautekeete ML, De-Bock RF, Van-Bockstaele DR, Colpin GC, Berneman ZN, Peetermans ME: Flow cytometric analysis of T-lymphocyte subpopulations in B-cell chronic lymphocytic leukemia: Correlation with clinical stage. Blut 55:447, 1987.
46. Orfao A, Gonzalez M, San Miguel JF, Cañizo MC, Galindo P, Caballero MD, Jimenez R, Lopez A: Clinical and immunological findings in large B-cell chronic lymphocytic leukemia. Clin Immunol Immunopathol 46:177, 1988.
47. Casares S, Rodriguez JM, Martin A, Parrado A: T-cell receptor gene rearrangements in lymphoid and non-lymphoid leukemias. Eur J Clin Invest 24:119, 1994.
48. Herndier BG, Kaplan LD, McGrath MS: Pathogenesis of AIDS lymphomas. AIDS 8:1025, 1994.
49. Baetz K, Isaaz S, Griffiths GM-CSF: Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. J Immunol 154:6122, 1995.